AFAMRL-TR-81-83
ADA 107614

COMPARATIVE BIOCHEMISTRY AND METABOLISM

Part 1: Carcinogenesis

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OCTOBER 1981

AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

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AIR FORCE/56780/3 November 1981 — 150

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFAMRL-TR-81-83	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* COMPARATIVE BIOCHEMISTRY AND METABOLISM: PART I: CARCINOGENESIS		5. TYPE OF REPORT & PERIOD COVERED Annual Report June 1980 - May 1981 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ronald C. Shank, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) F33615-80-C-0512
9. PERFORMING ORGANIZATION NAME AND ADDRESS The Regents of the University of California University of California, Irvine Irvine, California 92717		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62202F-6302-01-15
11. CONTROLLING OFFICE NAME AND ADDRESS Air Force Aerospace Medical Research Laboratory Aerospace Medical Division, Air Force Systems Wright-Patterson Air Force Base, Ohio 45433		12. REPORT DATE October 1981 13. NUMBER OF PAGES 38
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

APPROVED FOR PUBLIC RELEASE: DISTRIBUTION UNLIMITED

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Hydrazine
Hepatotoxins and DNA Methylation
DNA Methylation
7-Methylguanine
O^6-Methylguanine
Cytotoxicity and DNA Damage

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Oral administration of the inorganic hepatotoxin, hydrazine, to male Fischer 344 or Sprague Dawley rats results in the endogenous methylation of liver DNA at the 7- and O^6-positions of guanine. At doses below the LD_{50} (45, 60 or 75 mg hydrazine/kg body weight), methylation levels varied little and averaged about 500 μmol 7-methylguanine/mol guanine and 20 μmol O^6-methylguanine/mol guanine. At 90 mg hydrazine/kg body weight (approximately the 7-day LD_{50}) the methylation levels were 869 μmol 7-methylguanine and 58 μmol O^6-methylguanine/mol guanine 24 hours after toxicant administration. A single dose of 3 mg hydrazine/kg body

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weight did not result in detectable levels of methylguanines in liver DNA; however, after three or four daily administrations of hydrazine at this dose, liver damage was evident, and liver DNA contained 50-100 μmol 7-methylguanine/mol guanine, about the limit of analytical detection. Following a single oral administration of 90 mg hydrazine/kg body weight, liver DNA guanine rapidly became methylated. The time for half-maximum alkylation at 7-guanine was 30 minutes and at O^6-guanine, 45 minutes. The rates of removal of these methylated bases were consistent with published values from experiments using methylating carcinogens and with values obtained in this laboratory with the model compound, 1,2-dimethylhydrazine. Methylation of liver DNA was measured one hour after male Swiss Webster mice were given equimolar doses (0.33 mmol) of hydrazine or monomethylhydrazine to determine whether the organic compound was intermediate in the methylation of DNA in hydrazine-treated animals. This seemed not to be the case as approximately three-times more methylation of liver DNA guanine was observed in the hydrazine-treated animals compared to the monomethylhydrazine-treated mice. The methylation of liver DNA guanine was also seen in response to hepatotoxins in addition to hydrazine: carbon tetrachloride, ethanol, phosphorus, bromobenzene, and puromycin.

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism, Part 1: Carcinogenesis and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract Number F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1980 through May 1981.

R. C. Shank, Ph.D., was principal investigator for the subprogram. Acknowlegement is made to D. C. Herron, R. A. Becker, W. S. Bosan, M. J. Oldham, J. P. Reidy, and W. S. Pool for their significant research contributions and assistance in the preparation of this report. K. C. Back, Ph.D., Chief of the Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

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INTRODUCTION

Previous studies (Shank et al., 1980) provided some evidence to suggest that, at least in the case of the liver, weak carcinogens induce changes in DNA similar to the change seen in response to challenge by a strong carcinogen. The essential difference is that the weak carcinogen appears to bring about the change in DNA indirectly, when near toxic levels of the carcinogen are used; strong carcinogens, however, achieve the change in DNA directly and at doses an order of magnitude below toxic levels. It was shown that in rats and mice treated with a high dose of hydrazine, a hepatotoxin and weak carcinogen, the liver DNA rapidly underwent methylation of guanine residues (Barrows and Shank, 1978; 1980). The alkylation mechanism appeared to involve the endogenous methylation process which uses intracellular S-adenosylmethionine as the source of the methyl moiety. The methylation sites in liver DNA were the 7- and O^6-positions of guanine; the latter is a critical base-pairing site and its methylation has been shown, in a model system, to lead to base-pair transformation (mutation) (Gerchman and Ludlum, 1973).

The major activities of this subprogram focussed on the pharmacokinetics of DNA methylation in response to hydrazine administration and on determining whether other hepatotoxins stimulate the endogenous methylation of liver DNA guanine. Those studies are summarized in this report.

RESEARCH PROGRAM

METHODS DEVELOPMENT

Fractionation of DNA Hydrolysates by Liquid Chromatography

Several liquid chromatographic methods were tried in order to maximize the sensitivity in the detection of 7-methylguanine and O^6-methylguanine in DNA hydrolysates; all were simple modifications of the basic technique developed by Herron and Shank (1979). DNA was isolated and purified by the phenolic extraction method of Kirby (1962) as modified by Swann and Magee (1968). The nucleic acid was hydrolyzed in 0.1 M HCl (5 mg DNA/ml) for 30-40 minutes at 70°C to yield pyrimidine oligonucleotides and purines as free bases. DNA hydrolysates were fractionated on strong cation exchange columns by one of several high-pressure liquid chromatographic techniques (HPLC), the details of which are summarized in Table 1.

Each of these methods permitted the detection of 80-100 μmol 7-methylguanine/mol guanine and approximately 10 μmol O^6-methylguanine/mol guanine in the single analysis of 1 or 5 mg hydrolyzed DNA applied to an analytical or preparative column, respectively.

TABLE 1. PARAMETERS OF LIQUID CHROMATOGRAPHIC TECHNIQUES

Methods	Column[1]	Mobile phase system[2]	Flow (ml/min)	Detector[3]
1	50 cm prep	A for 20 min, then B	4	Fluor.
2	50 cm prep	C, isocratic	4	uv
3	50 cm prep	A for 12 min, then B	4	uv
4	50 cm prep	B, isocratic	4	uv
5	50 cm prep	A for 12 min, B for 28 min, then D	4	Fluor.
6	Analytical	A for 1 min, then B	2	Fluor.

[1]Whatman prepacked strong cation exchange columns; 50 cm preparative Partisil 10 Magnum 9; 25 cm analytical Partisil 10.

[2]A, distilled water; B, 0.1 M $NH_4H_2PO_4$ pH 2.0; C, 0.035 M HCl; D, 0.1 M ammonium acetate, pH 4.5.

[3]Fluor., fluorescence at 286 nm with interference emission filter at 344 nm; uv, ultraviolet absorbance at 275 nm.

A new hydrolysis technique was developed to permit the analysis of larger amounts of DNA in a single attempt. 7-Alkylguanine is known to depurinate spontaneously from DNA heated in neutral solution (Lawley and Brookes, 1963), while O^6-methylguanine does not. Up to 100 mg DNA was dissolved in 10 mM sodium cacodylate pH 7.0 (5 mg DNA/ml buffer) and heated to 100°C for 35 minutes to release 7-methylguanine from the DNA polymer; the partially apurinic DNA was precipitated from the neutral hydrolysate by adding 0.11 volume 1 M HCl and hydrolyzed as above. Neutral hydrolysates containing 7-methylguanine and small amount of guanine and adenine were fractionated on 25-cm preparative Partisil 10 Magnum 9 strong cation exchange columns (Whatman, Clinton, NJ) with a two-step gradient of water for 4 minutes and then 0.1M $NH_4H_2PO_4$ pH 2.0 at a flowrate of 4 ml/minute; hydrolysate representing as much as 50 mg DNA could be applied to a column in a single injection. Hydrolysates of partially apurinic DNA were fractionated on 25-cm analytical Partisil 10 strong cation exchange columns isocratically with 0.1 M $NH_4H_2PO_4$ pH 2.0 at 2 ml/minute. O^6-Methylguanine could be resolved quantitatively from as much as 5 mg hydrolyzed DNA by this technique. In both cases elution of bases was monitored by fluorescence spectrophotometry at an excitation wavelength of 286 nm and using an interface emission filter at 344 nm. Quantitation was achieved using a Hewlett Packard 3380S electronic reporting integrator which had been calibrated by injecting known quantities of authentic guanine, 7-methylguanine, and O^6-methylguanine onto the column.

Partial Hepatectomies in the Rat

As part of the study on the relation between DNA synthesis and DNA methylation in hydrazine toxicity, it was desired to stimulate DNA synthesis in adult rat liver. This stimulation can readily be achieved by surgical removal of 50-60% of the liver; in partial hepatectomized rats the liver begins regeneration and DNA synthesis reaches maximum activity approximately 24 hours after the surgery. Higgins and Anderson (1931) demonstrated that adult rats could well tolerate surgical removal of both the median and left lateral lobes, representing approximately 70% of the hepatic mass. Twenty-four hours after surgery the remaining liver tissue has increased approximately 36%; the liver completely regenerates in 2-3 weeks.

A rat was anesthetized with ether; a median-line incision was made 3-4 cm posteriorly from the xiphoid process of the sternum, and the median and left lateral lobes of the liver were externalized, ligated, and excised. The abdomen was closed in two layers, using wound clips. No special postoperative care was needed. Attempts to remove only the median lobe of the liver usually resulted in impaired blood supply to the left lateral lobe and tissue ischemia. Twenty-four hours after surgery, the remaining liver lobes were pale, enlarged, and rounded, with loss of the characteristic sharp edges of each lobe.

HYDRAZINE AND METHYLATION OF LIVER DNA GUANINE

Methylation of Liver Guanine in the Sprague Dawley Rat Following Hydrazine Administation

Previously, all studies on hydrazine and DNA methylation were done using the Fischer 344 rat (Shank et al., 1980), the strain used in the inhalation carcinogenicity study of MacEwen et al. (1979). For economic reasons, the DNA methylation response to hydrazine administration was investigated in the Sprague Dawley rat to determine whether this strain could be used as an alternate to the Fischer 344.

Two young male Sprague Dawley rats (Simonsen Labs, Gilroy, CA) were given orally 90 mg hydrazine/kg body weight; four rats were given 0.1 M HCl only. All animals were decapitated 24 hours later and liver DNA was isolated, purified, and hydrolyzed in 0.1 M HCl. The hydrolysate was fractionated by preparative liquid chromatography (Table 1, Method 1). No methylated guanine was detected in 18.5 mg DNA from control rats; analysis of 12.0 mg DNA from hydrazine-treated rats contained 604 µmol 7-methylguanine and 48 µumol O^6-methylguanine per mol guanine (Figure 1) which is similar to the levels seen in liver DNA following hydrazine administration to Fischer 344 rats

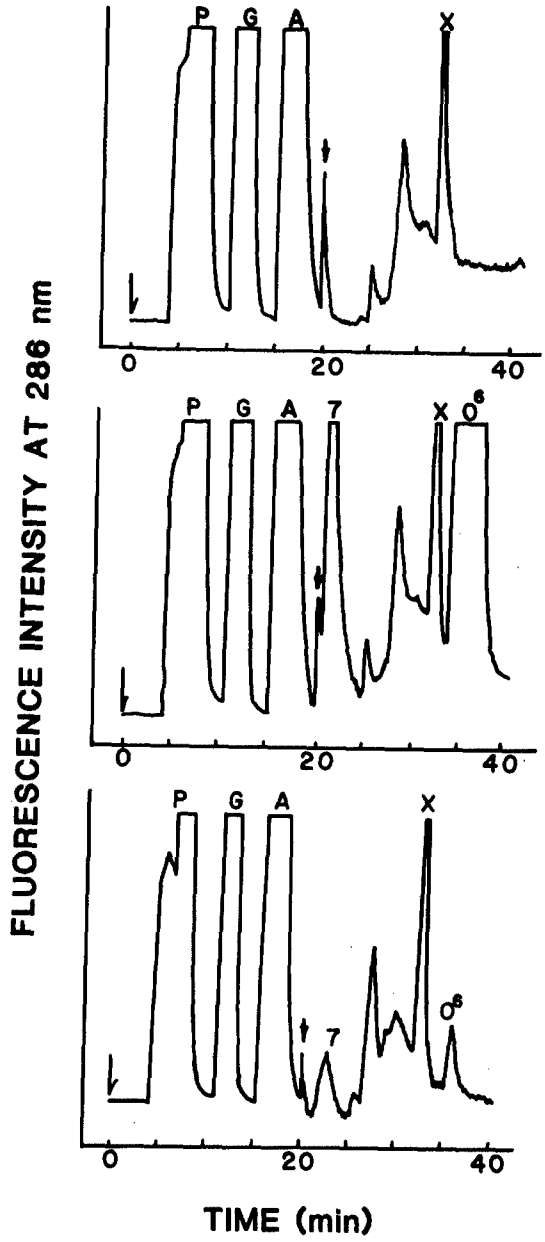

Figure 1. Elution profiles of whole hydrolysate of liver DNA prepared from control and hydrazine-treated rats. DNA was hydrolyzed in 0.1 M HCl and 1 ml hydrolysate (5 mg DNA) was fractionated according to HPLC Method 1. Injection order of hydrolysates onto the column was: (1) DNA hydrolysate from rats given orally 90 mg hydrazine/kg body weight and decapitated 24 hours later (bottom panel); (2) DNA hydrolysate from rats given 0.1 M HCl orally (control) and decapitated 24 hours later (top panel); (3) DNA hydrolysate from control animals, to which was added standard 7-methylguanine and O^6-methylguanine (middle panel). P, pyrimidine oligonucleotides; G, guanine, A, adenine; X, contaminant; 7, 7-methylguanine; O^6, O^6-methylguanine; half arrow, time hydrolysate injected onto column; full arrow, 10-fold increase in sensitivity on fluoresence detector and change of mobile phase from water to phosphate buffer. Contaminant is an unidentified material which presumptive evidence suggests was derived from the 2-ethoxyethanol used to precipitate the DNA in the isolation procedure.

(7-methylguanine, 799 µmol/mol guanine; O^6-methylguanine, 52 µmol/mol guanine). It was concluded that the Sprague Dawley rat is an effective substitute for the Fischer 344 rat in these investigations.

Effect of Hydrazine Dose on DNA Methylation

Young male Sprague Dawley rats were fasted overnight and given orally 0, 45, 60, 75, or 90 mg hydrazine/kg body weight in 0.1 M HCl; there were 4 animals per dose. Twenty-four hours after treatment the animals were decapitated and livers were excised and rinsed in ice cold saline. A small wedge of each liver was fixed in 10% neutral buffered formalin. The remaining liver was frozen at -70°C for later isolation of DNA.

DNA was subjected to a neutral hydrolysis by heating DNA dissolved in 10 mM sodium cacodylate pH 7.0 (5 mg DNA per ml buffer) at 100°C for 35 minutes. The partially apurinic DNA was precipitated from the neutral hydrolysate by adding 0.11 volume 1 M HCl. The neutral hydrolysate was evaporated to dryness in a rotary vacuum evaporator at 40°C and redissolved in 0.5 ml 0.1 M ammonium phosphate pH 2.0 with 5 minutes sonication; the evaporation flask was rinsed and sonicated again with 0.5 ml phosphate buffer for quantitative transfer of the neutral hydrolysate to a storage vial.

The precipitated partially apurinic DNA was hydrolyzed in 0.1 M HCl at 70°C for 45 minutes, filtered (0.65 μm pore size) before analysis by high pressure liquid chromatography.

Neutral hydrolysate dissolved in 0.1 M ammonium phosphate pH 2.0 was fractionated on a Whatman preparative strong cation exchange column using a two-step mobile phase system of water for the first 4 minutes followed by 0.1 M ammonium phosphate pH 2.0, both phases pumped at 5 ml/minute. Under these conditions guanine eluted at approximately 10 minutes, adenine at 14 minutes, and 7-methylguanine at 17 minutes. The results are summarized in Table 2.

These results agree with those from the early studies in which DNA methylation was detected using radiolabeled methionine (Shank et al., 1980). At the lower doses the extent of methylation varied little, but when the dose was near the LD_{50}, the methylation levels increased sharply.

The histology slides were examined by Paul M. Newberne, D.V.M., Ph.D., Professor of Nutritional Pathology at the Massachusetts Institute of Technology. No histological changes could be associated with the hydrazine treatment; no explanation was apparent.

Liver DNA Methylation with Multiple Exposures to Hydrazine

A carcinogenic regimen for the induction of liver cancer with hydrazine requires multiple exposures to the hepatotoxin. It is thus important to determine the DNA methylation pattern in rat liver

TABLE 2. METHYLATION OF LIVER DNA 24 HOURS AFTER ADMINISTRATION OF VARIOUS DOSES OF HYDRAZINE TO RATS

Dose, mg/kg	µmol 7-MeG/mol G	µmol O^6-MeG/mol G
0	nd[1]	nd[2]
45	419	14
	422	16
	mean = 421	mean = 15
60	398	12
	724	29
	mean = 561	mean = 20
75	564	19
	498	25
	mean = 531	mean = 22
90	794	48
	944	68
	mean = 869	mean = 58

[1]no 7-methylguanine detected (less than 50 µmol 7-MeG/mol G)
[2]no O^6-methylguanine detected (less than 2 µmol of O^6-MeG/mol G)

following multiple exposures to hydrazine. As the first step in a study on the effect of multiple exposures to hydrazine, 8 male Fischer 344 rats (173-200 g) were given 3 mg hydrazine/kg body weight in 0.1 M HCl by intubation daily for 1, 2, 3, or 4 days; 2 rats were decapitated 12 hours after 1, 2, 3, or 4 administrations of hydrazine; this dose regimen caused a 6.6% loss in body weight over the 4-day period. DNA was isolated from each individual liver and was analyzed for the presence of methylated bases using fluorescence high pressure liquid chromatography (Table 1, Method 5).

The results are summarized in Figure 2 which gives elution profiles for liver DNA hydrolysates prepared from animals given one, two, three, or four daily doses of 3 mg hydrazine/kg body weight (single oral LD_{50} is approximately 80 mg/kg). Approximately the same amount of hydrolyzed DNA (4-6 mg) is represented in each profile.

From this experiment it is apparent that multiple low doses of hydrazine to rats are capable of eliciting the DNA methylation response; a small amount of 7-methylguanine (50-100 µmol/mol guanine) was readily detectable in liver DNA by the third or fourth exposure to hydrazine. O^6-Methylguanine was not observed in this experiment; in the past, this methylated purine has always occurred at

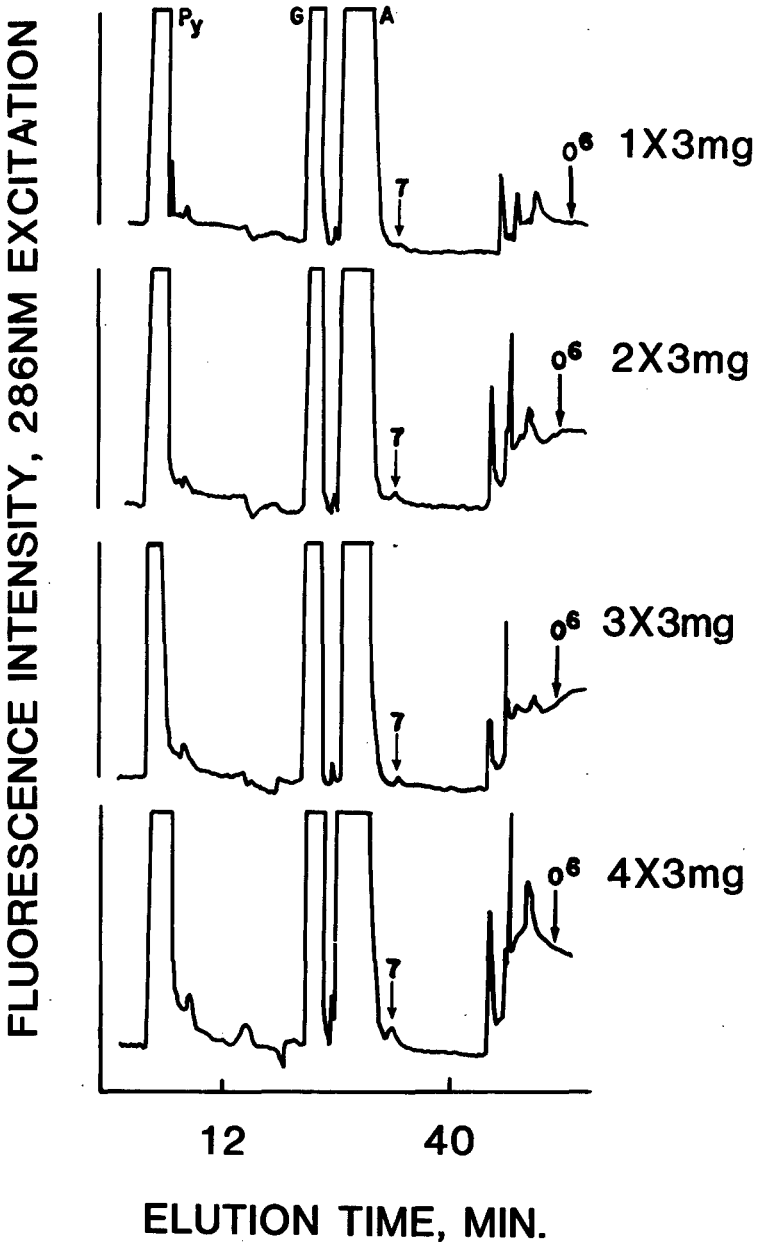

Figure 2. Appearance of 7-methylguanine in rat liver DNA after multiple exposures to 3 mg hydrazine/kg body weight. Elution profiles of DNA hydrolysates (4-6 mg DNA) from rats killed 12 hr after treatment with 1-4 single daily doses of hydrazine. Arrows denote elution position of 7-methylguanine (7) and O^6-methylguanine (O^6). Py, pyrimidine oligonucleotides; G, guanine, A, adenine.

concentrations 10-30 times below those of 7-methylguanine and may have been present at a concentration below the limit of detection (10 μmol/mol guanine in this sytem). Twelve hours after the third daily injection of hydrazine, liver damage was evidence by a swollen, pale, greasy appearance. Further studies on the response to multiple low level exposures to hydrazine are necessary to determine whether

detection of 7-methylguanine after three daily injections was due to accumulation of this base over the three-day exposure period or due directly to liver damage, if indeed 7-methylguanine accumulation and cytotoxicity are separable.

Rates of Formation and Removal of Methylguanines from Liver DNA of Rats Given Hydrazine

In an earlier study, it was shown that liver DNA methylation occurred rapidly after hydrazine administration (Shank et al., 1980). That experiment used radiolabeled methionine to permit detection of methylated bases; the study was repeated and expanded, using the quantitative fluorescence technique. The experiment was designed to measure how rapidly liver DNA methylation occurs after administration of hydrazine, and how long that methylation (7-methylguanine and O^6-methylguanine) persists. It was important to determine whether 7-methylguanine in DNA of poisoned animals was lost rapidly and simultaneously with cell lysis (sharp loss of cells around 9 hours post-treatment) or lost slowly due to spontaneous depurination in surviving cells (half-life of 7-methylguanine in DNA is 45-48 hours, see below). Since it was felt that the aberrant methylation seen in hydrazine-treated rats was associated with hepatotoxicity and related to the carcinogenicity of hydrazine, it was important to determine that all the cells which suffered the DNA methylation were not killed, thus precluding the possibility for somatic mutation.

Fasted male Fischer rats (200-250 g body weight) were given by intubation 90 mg hydrazine/kg body weight in 0.1 M HCl; control animals were given vehicle only. Animals which were scheduled to survive longer than 24 hours were given food six hours after toxicant administration. Pairs of animals were decapitated at 0, 0.25, 0.50, 1, 6, 12, 24, 48, 72 and 96 hours after toxicant administration and their livers excised and frozen at -70°C. Thin sections of each liver were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin for histopathologic analysis.

DNA was analyzed for the presence of 7-methylguanine and O^6-methylguanine by the same hydrolytic and chromatographic techniques as described above for the dose-response study.

Partially apurinic DNA hydrolysate was fractionated on a Whatman analytical strong cation exchange column with an isocratic mobile phase of 0.1 M ammonium phosphate pH 2.0 at 2 ml/minute. Under these conditions guanine eluted at approximately 2 minutes, adenine at 2.9 minutes and O^6-methylguanine at 11.5 minutes; a contaminant eluted at 8.5 minutes.

The results are summarized in Figures 3 and 4. It is clear that both 7-methylguanine and O^6-methylguanine can be detected in liver DNA as early as 15 minutes after oral hydrazine administration (Figure 3). The time to half maximum methylation was approximately 30 minutes for 7-methylguanine and 45 for O^6-methylguanine.

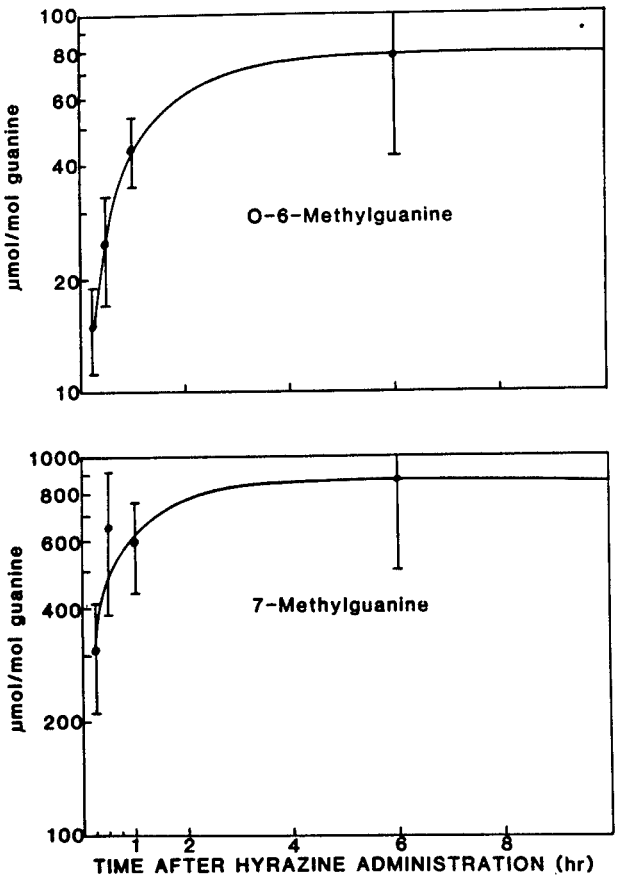

Figure 3. Formation of 7-methylguanine and O^6-methylguanine in liver DNA following oral administration of 90 mg hydrazine/kg body weight to rats. Data represent means with range of two indpendent determinations.

The DNA methylation remained at maximum levels from 6 to 24 hours after hydrazine administration, and then the methylated guanines were removed from liver DNA at rates consistent with published values for the chemical depurination of 7-methylguanine (50% removal in 47 hours) and the enzymatic removal of O^6-methylguanine (50% removal in 13 hours) (Figure 4). These results can be interpreted as further evidence for the confirmation of identity of these bases in DNA.

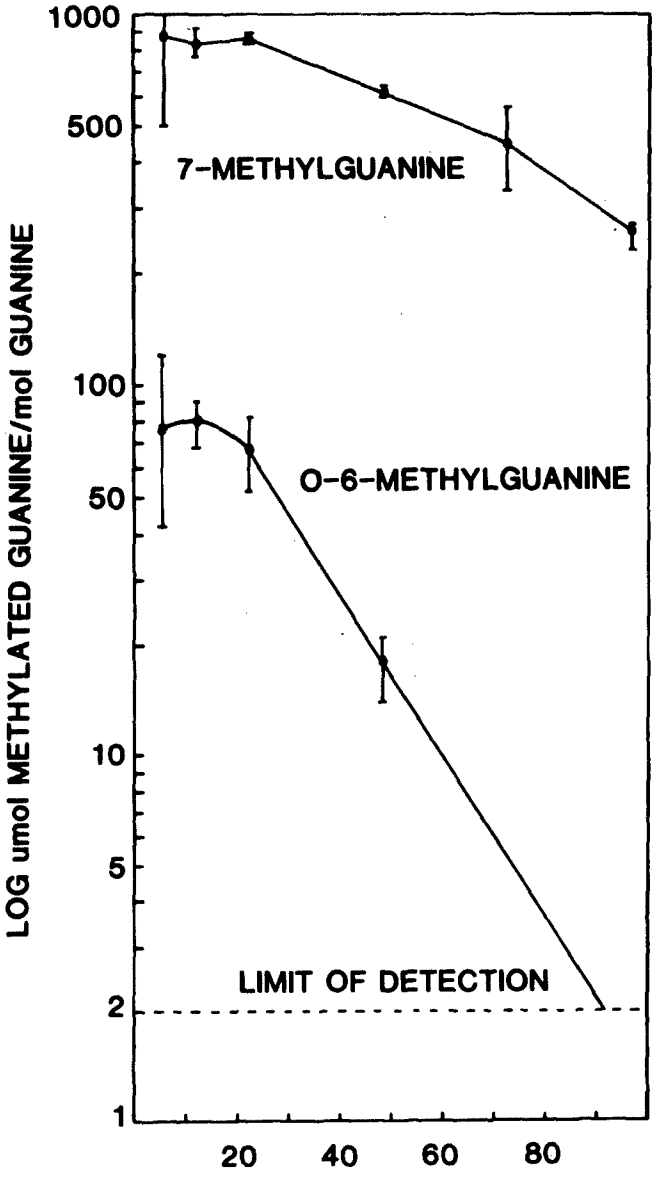

Figure 4. Removal of 7-methylguanine and O^6-methylguanine from liver DNA following oral administration of 90 mg hydrazine/kg body weight to rats. Data represent means with range of two independent determinations.

Professor Newberne of the Massachusetts Institute of Technology examined the liver specimens from this experiment. He reported (T=time in hours after hydrazine administration):

T = 0 normal
T = 0.25 minimal hydropic change in the periportal area and an occasional cell with a pyknotic nucleus

T = 0.50 a moderate periportal hydropic change with pyknosis, vesicular nuclei, chromatin clumping and an occasional prominent nucleolus

T = 1 periportal hydropic change with pyknosis and single cell necrosis

T = 6 periportal hydropic change extending out into the midzonal nuclear membrane. There were also foci of hemorrhagic necrosis with the nuclei at the periphery of such foci markedly vesicular with prominent chromatin clumps and margination of the chromatin. The necrotic area contained cells with pyknosis, karyorrhexis, and infiltration of polymorphonuclear leukocytes. The hepatocyte nuclei varied some in size with an occasional megalocyte. The nuclear chromatin was marginated, lobulated and with strands extending from the clumps to the nuclear membrane.

T = 12 periportal hydropic change involving three-fourths or more of the lobule. The hepatocytes contain prominent nucleoli. There was chromatin margination and clumping with enlarged regular sized vesicular nuclei.

T = 24 periportal hydropic change involving about two-thirds of the lobule. In addition there was periportal edema of the connective tissue and bile duct supporting stroma, polymorphonuclear infiltration, scattered pyknosis and enlarged vesicular nuclei.

T = 48 periportal hydropic change occupying or involving at least one-half of the lobule. In addition there was scattered pyknosis, double nuclear hepatocytes, necrotic hepatocytes but apparently more uniformitive to the size and shape of the hepatocytes.

T = 72 periportal hydropic change persisting in one-half of the lobule with periportal edema. The hepatocytes had nuclear chromatin clumps, margination of the chromatin, hyperchromatic hepatocytes and some with double nuclei.

T = 96 periportal to midzonal hydropic change with pyknosis persisting and single cell necrosis. Many of the Kupffer cells contained fragments of nuclear chromatin indicating a persistent necrosis of parenchymal cells. This last slide seemed to indicate that although there was a change in the methylation of DNA the morphologic picture had not been reversed even after 96 hours. It is likely that this requires a somewhat longer period of time.

Several laboratories have measured the rates of removal of 7-methylguanine and O^6-methylguanine from liver DNA after treatment of rats with varying doses of methylating agents; all the previous investigations relied on administration of radiolabeled carcinogens to methylate the DNA. As the determination of the extent of DNA methylation in the current studies relied on optical rather than

radiolabel techniques, it was decided to determine the rates of removal of 7-methylguanine and O^6-methylguanine from liver DNA of rats treated with the potent methylating carcinogen, 1,2-dimethylhydrazine, using the optical (fluorescence) method. The study has been published (Herron and Shank, 1981).

Unfasted male Sprague Dawley rats (100-150 g body weight) were injected intraperitoneally with a single dose of 10.2, 40.7, 81.5 or 163 mg 1,2-dimethylhydrazine/kg body weight (as a neutral aqueous solution) and were decapitated 3, 6, 12, 24, 48, 72, 96 or 120 (highest dose only) hours later. DNA was isolated from individual livers (from 6 animals per dose per interval) and analyzed for the presence of 7-methylguanine and O^6-methylguanine by the liquid chromatographic method described above for analysis of partially apurinic DNA.

A pharmacokinetic analysis of the decline in methylguanine levels with time was done, assuming the removal rates followed first order kinetics. The logarithm of the methylguanine concentration in liver DNA was plotted against the time after maximum alkylation levels had been reached (Figures 5 and 6). The half-lives of 7-methylguanine and O^6-methylguanine in liver DNA were calculated from those linear plots and are summarized in Table 3.

These values, determined in our own laboratory under the same conditions used to study DNA methylation in hydrazine-treated

TABLE 3. HALF-LIVES OF METHYLATED GUANINES IN LIVER DNA

Dose (SDMH) mg/kg body weight	$T_{\frac{1}{2}}$ 7-Methylguanine	O^6-Methylguanine[1]
10.2	48	13
40.7	47	16, 37
81.5	45	19, 50
163	45	17, 46

[1] $T_{\frac{1}{2}}$ separable into two components on basis of amount of O^6-methylguanine remaining in DNA; at concentrations of O^6-methylguanine above 400 µmol/mol guanine, the rapid rate of removal (shorter $T_{\frac{1}{2}}$) dominated, while at concentrations of O^6-methylguanine below 300 µmol/mol guanine, the slower rate of removal (longer $T_{\frac{1}{2}}$) was apparent.

animals, were used as the comparators for the rates of removal of these methylguanines from liver DNA in the hydrazine experiments. As stated above, the rates of removal in the two studies were in close agreement.

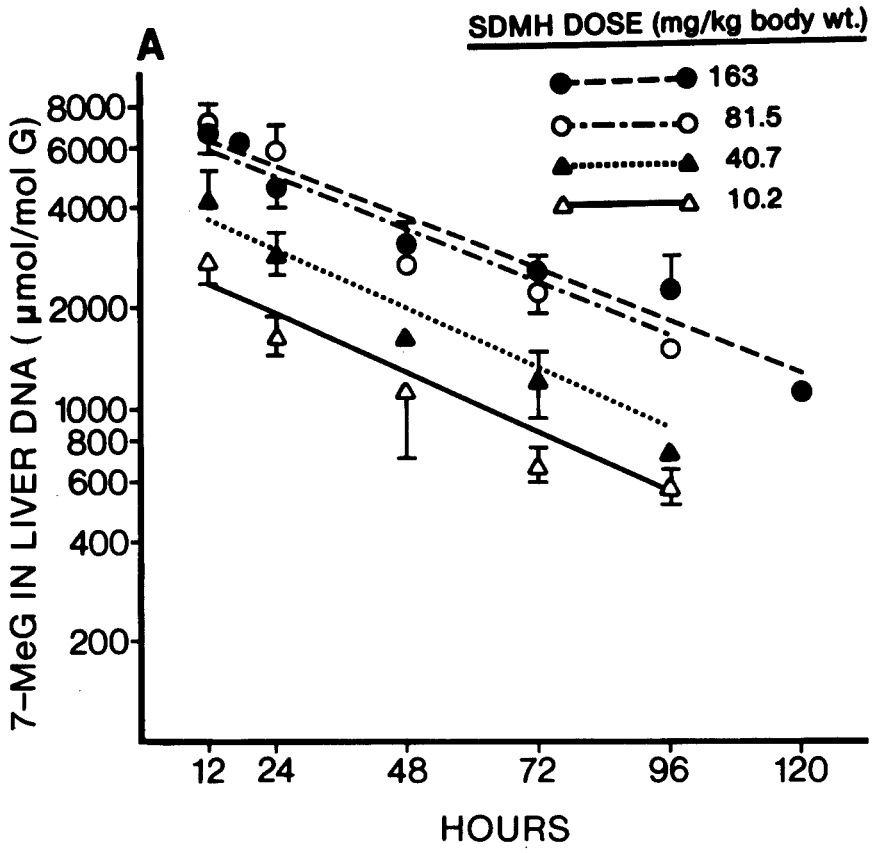

Figure 5. Removal of 7-methylguanine from liver DNA following intraperitoneal administration of 1,2-dimethylhydrazine (SDMH) to rats.

Effect of Ethionine Pretreatment on DNA Methylation in Hydrazine Poisoned Rats

The methionine analog, ethionine, can deplete S-adenosylmethionine pools and inhibit DNA methyltransferase. The results of the studies on DNA methylation in hydrazine-treated animals suggest that the methylation process is mediated by S-adenosylmethionine and may be catalyzed by a DNA methyltransferase. A previous experiment was carried out to determine whether ethionine could block DNA methylation in hydrazine-treated rats (Shank et al., 1980), and that experiment was repeated here with only slight modification to see if the earlier results could be confirmed.

Four fasted Fischer 344 male rats (190-195 g) were given intraperitoneally 300 µCi ^{3}H-methyl-methionine to label the S-adenosyl-

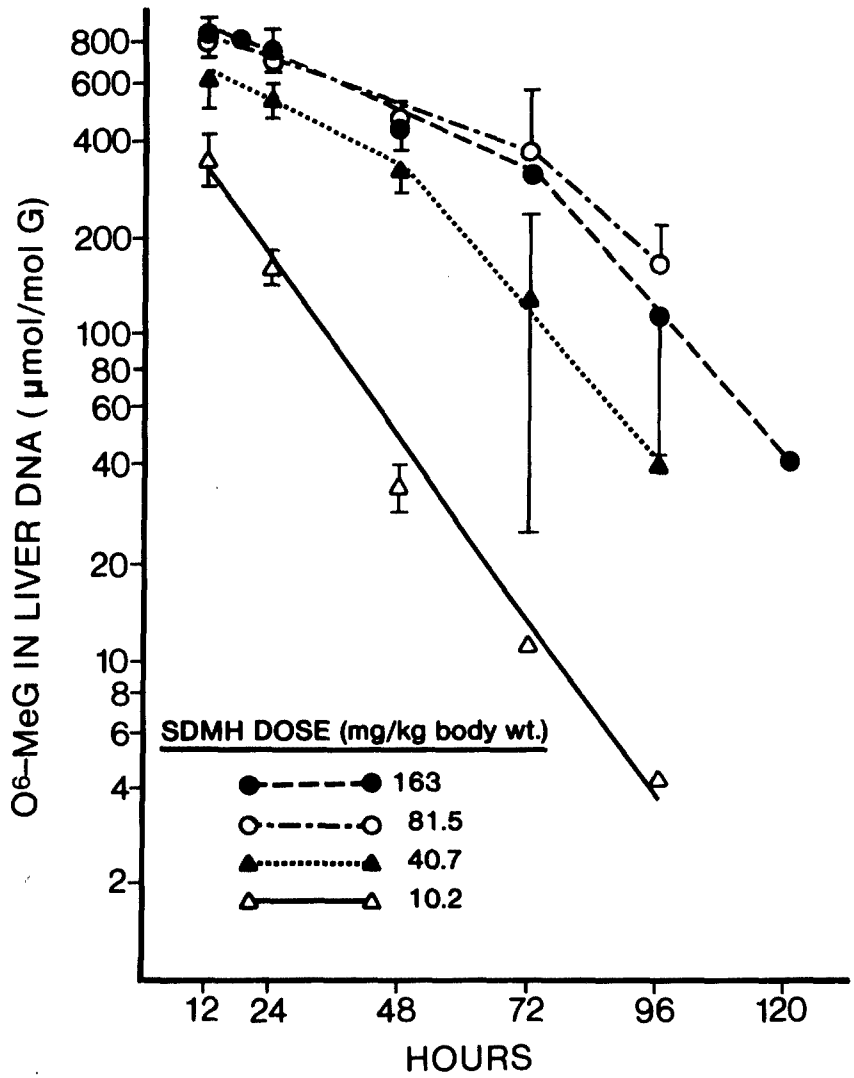

Figure 6. Removal of O^6-methylguanine from liver DNA following intraperitoneal administration of 1,2-dimethylhydrazine (SDMH) to rats.

methionine pools; 15 minutes later two rats were given 500 mg ethionine/kg body weight intraperitoneally (saline was given to controls); 15 minutes later all four rats were given orally 60 mg hydrazine/kg body weight and killed 3 hours later. Liver DNA was analyzed for methylated bases using a reverse phase liquid chromatographic system: Whatman ODS-2 preparative 50 cm column, water-methanol gradient of 10-100% methanol developed over 60 minutes with a concave curvature of N = 2.5 at a flow rate of 4 ml/minute; pH of the water was adjusted to 4.0 with glacial acetic acid before gradient preparation; elution order was pyrimidine oligonucleotides,

guanine, 7-methylguanine, adenine, O^6-methylguanine. The results are summarized in Table 4. Pretreatment of animals with ethionine shortly before hydrazine administration effectively blocked the formation of N-methyltransferase in rat liver DNA and also appeared to increase the incorporation of the methyl group from methionine into the pyrimidine oligonucleotide fraction, presumably representing increased formation or stability of 5-methylcytosine in DNA. These results then confirm those of the earlier study (Shank et al., 1980).

TABLE 4. EFFECT OF ETHIONINE ON DNA METHYLATION IN HYDRAZINE-TREATED RATS

Fraction	Amount Base (dpm/μmol guanine) Control	Ethionine
Pyrimidine oligonucleotide	177	247
Guanine	16	39
Trough	nd	28
7-Methylguanine	36	20
Adenine	9	25
Trough	-	1
O^6-Methylguanine	8	6
Post-O^6-methylguanine	4	2

nd = none detected
- = not analyzed

Methylation of Liver DNA Guanine in Mice Given Hydrazine or Monomethylhydrazine

The involvement of S-adenosylmethionine in the methylation of liver DNA following administration of an inorganic hepatotoxin, such as hydrazine, suggests the toxicant could be methylated by S-adenosylmethionine to form monomethylhydrazine, the administration of which is already known to result in the methylation of liver DNA. Hawks and Magee (1974) reported a level of 63 μmol 7-methylguanine/mol guanine in female NMRI mice 6 hours after receiving 15 mg ^{14}C-monomethylhydrazine/kg body weight subcutaneously; O^6-methylguanine levels were not determined.

Eleven male Swiss Webster mice (27-41 g) were fasted overnight and then given orally 15 mg (0.33 mmol) monomethylhydrazine/kg body weight; similarly, 11 mice were given an equimolar dose (10.4 mg/kg body weight) of hydrazine. The animals were decapitated one hour after treatment. Levels of methylated guanines were determined in neutral thermal hydrolysates of whole DNA and mild acid hydrolysates of partially apurinic DNA (23.9 mg DNA from monomethylhydrazine-treated mice and 24.6 mg from hydrazine-treated mice). The estimates

for the amounts of 7-methylguanine and O^6-methylguanine in the liver DNA of the treated mice are summarized in Table 5. Administration of equimolar doses of the two compounds resulted in considerably more DNA methylation in hydrazine-treated animals; also the molar ratios of 7-methylguanine to O^6-methylguanine were not the same in the two cases, implying that the methylating agent is not the same for hydrazine and monomethylhydrazine. These results suggest that monomethylhydrazine is not an important intermediate in the methylation of liver DNA in hydrazine-treated animals.

TABLE 5. LIVER DNA METHYLATION IN MICE GIVEN EQUIMOLAR DOSES OF MONOMETHYLHYDRAZINE AND HYDRAZINE

Treatment	DNA Methylation (μmol/mol guanine) 7-methylguanine	O^6-methylguanine
Monomethylhydrazine	52	2
Hydrazine	157	9

VARIOUS HEPATOTOXINS AND METHYLATION OF LIVER DNA

In earlier work (Shank et al., 1980) an experiment was carried out to determine whether the observed methylation of DNA was a response specific to hydrazine administration or a nonspecific response to chemical insult to the liver; this distinction was suggested by results from an unrelated study by Ruchirawat (1974), who found two sources of the methyl moiety in liver DNA 7-methylguanine following administration of the hepatotoxin and hepatocarcinogen, dimethylnitrosamine and a minor source was methionine, not involving dimethylnitrosamine-labeling of the one-carbon pool. The hepatoxins studied by Shank and coworkers (1980) included carbon tetrachloride and thioacetamide. The present study examined, in addition, ethanol, phosphorus, bromobenzene, and puromycin.

DNA Guanine Methylation in Fischer Rats Given Hydrazine, Carbon Tetrachloride, Ethanol, or Thioacetamide

Four male rats (165-175 g) were given 60 mg hydrazine/kg body weight in 0.1 M HCl; another 4 rats (137-160 g) were given 1 g CCl_4/kg body weight in corn oil; a third group of 4 rats (160-178 g) were given 5 g ethanol/kg body weight as 70% ethanol in water; 4 rats (160-178 g) were given 200 mg thioacetamide/kg body weight in water; 5 control animals (152-180 g) received 0.1 ml water. All administrations were made by stomach tube to fasted rats, and all animals were decapitated 12 hours after treatment. DNA was isolated from liver of animal pairs and hydrolyzed in 0.1 M HCl. The hydrolysates were examined by liquid chromatography Method 5 (Table 1).

O^6-Methylguanine fractions were lyophilized and redissolved in 0.1 M HCl and rechromatographed using Method 6 (Table 1). The results are summarized in Table 6.

TABLE 6. METHYLGUANINES IN LIVER DNA OF RATS GIVEN VARIOUS HEPATOTOXINS AND KILLED 12 HOURS LATER

Hepatotoxin	Amount DNA analyzed (mg)	Amount methylated base (μmol/mol guanine) 7-methylguanine	O^6-methylguanine
Control	10	nd	nd
Hydrazine	9	193	22
Carbon Tetrachloride	4	560	14
Ethanol	4	398	nd
Thioacetamide	7	nd	nd

DNA Guanine Methylation in Sprague Dawley Rats Given Carbon Tetrachloride, Ethanol, Phosphorus, Bromobenzene or Puromycin

Fasted male Sprague Dawley rats (220-250 g) were given on a per kg body weight bases, 1 g CCl_4 in corn oil, 5 g ethanol as 70% ethanol in water, 8 mg yellow phosphorus as a 0.5% solution in olive oil, 750 mg bromobenzene in corn oil, or 20 mg puromycin in 0.04 M NaH_2PO_4-Na_2HPO_4 pH 7.4 in 0.154 M NaCl; control animals received appropriate amounts of corn oil, water, or phosphate buffer. All administrations were made by stomach tube except that for puromycin which was given in 10 equal doses (10 x 2 mg/kg body weight) every 90 minutes by intraperitoneal injection; the control animals were treated similarly. The animals were decapitated 12-24 hours later. Liver DNA was first hydrolyzed by the neutral thermal technique and then the resultant partially apurinic DNA was hydrolyzed in 0.1 N HCl. The hydrolysates were analyzed quantitatively for 7-methylguanine and O^6-methylguanine by liquid chromatography. The results are summarized in Table 7; O^6-methylguanine was not detected in any of the samples.

A total of seven hepatotoxins have been examined for their capacity to stimulate endogenous methylation of liver DNA, presumably via the intermediate of S-adenosylmethionine. Three of these hepatotoxins, hydrazine, carbon tetrachloride and phosphorus, cause both a fatty liver and necrosis; two compounds, bromobenzene and thioacetamide, produce necrosis without a fatty liver. Ethanol and puromycin produce a fatty liver without necrosis. No correlation, then, can yet be made between DNA methylation and either type of hepatotoxin; apparently all hepatotoxins stimulate this methylation of liver DNA to some extent.

TABLE 7. 7-METHYLGUANINE IN LIVER DNA OF RATS GIVEN CARBON TETRACHLORIDE, ETHANOL, PHOSPHORUS, BROMOBENZENE, OR PUROMYCIN

Agent	Kill time (hour)	Amount DNA analyzed (mg)	Amount 7-methylguanine (μmol/mol guanine)
Oil	12	--	--
Oil	24	13.0	nd
Water	12	--	--
Buffer	16	--	--
Carbon Tetrachloride	12	9.6	nd
		14.8	nd
Ethanol	12	18.9	23
		19.0	28
Phosphorus	24	18.4	62
		22.6	81
Bromobenzene	24	16.1	55
		10.6	nd
Puromycin	16	25.0	110
		14.0	nd

limits of detection: 7-methylguanine, ca 20 μmol/mol guanine; O^6-methylguanine, 1 μmol/mol guanine

The difficulty in gaining reproducibility in the studies with various hepatotoxins appears to be the technical problem of limits of detection in the analytical technique. The DNA hydrazine levels are quite low compared to those usually encountered in analyses of DNA from animals treated with methylating agents. As the limits of detection improve and as the dose and time relationships are elucidated, greater reproducibility will be expected.

SUMMARY AND CONCLUSIONS

Since most chemical carcinogens which have been studied by the biochemist and toxicologist have been shown to form adducts with DNA presumably as the first step in initiation, it was decided in the Air Force studies to determine the nature of DNA adducts, if any, in hydrazine-treated animals. The most likely DNA adducts to form would be hydrazino derivatives where hydrazine would react directly with primary amino groups on the bases, or methylated bases that could derive from methylation of hydrazine in the liver by N-methyltransferases to form monomethylhydrazine; Hawks and Magee (1974) have shown that monomethylhydrazine is metabolized to a methylating intermediate in the mouse. The first studies by the principal investigator began with an investigation into the methylation of liver DNA in hydrazine-treated animals.

Rats and mice were given single oral doses of hydrazine which were cytotoxic but seldom lethal. Simultaneously, the animals were given intraperitoneally (^{14}C- or ^{3}H-methyl)methionine to label the S-adenosylmethionine pool; the methionine injections were repeated every hour until the animals were killed in order to keep the S-adenosylmethionine pool labeled. Liver DNA was isolated, purified, and hydrolyzed in dilute acid to release free purine bases. Carrier 7-methylguanine and O^6-methylguanine were added to the hydrolysate which was then fractionated by high pressure liquid chromatography. Radioactive material co-eluted with standard methylated guanines, suggesting that the DNA had been methylated; since hydrazine is inorganic, the methyl source was presumed to be S-adenosylmethionine. This presumption was further supported by experiments which showed that ethionine was an effective inhibitor of the formation of the methylated guanines in the liver DNA (Barrows and Shank, 1978; Barrows and Shank, 1980; Shank and Barrows, 1981).

Using preparative liquid chromatography sufficient quantities of the putative 7-methylguanine were isolated and purified for spectral analysis; the suspect material was shown to have ultraviolet spectra in acid and alkaline solution that closely followed those for authentic standard 7-methylguanine (Shank and Barrows, 1981). The suspect material was also shown to have chromatographic properties identical to standard 7-methylguanine in five liquid chromatographic systems (both cation exchange and reverse phase chromatography) and in a paper chromatographic technique (Barrows and Shank, 1978). A study is in progress for the mass spectral analysis of the putative 7-methylguanine to confirm identity. The identity of O^6-methylguanine has been supported by similar chromatographic evidence, and the observation that acidic thermal hydrolysis of the suspect compound yields material which is chromatographically identical to guanine. Liver DNA from hydrazine-treated animals is currently being analyzed by radioimmunoassay for O^6-methylguanine by Dr. Peter F. Swann of the Courtauld Institute for Biochemistry at Middlesex Hospital in London.

The S-adenosylmethionine pool in liver is highly dynamic; a pulse of labeled methionine results in maximal specific activity of S-adenosylmethionine in 10-15 minutes with no further detectable radioactivity in S-adenosylmethionine 60 min after methionine injection (Craddock, 1974). In the hydrazine studies labeled methionine was administered simultaneously with hydrazine and hourly thereafter until the animals were killed. This protocol was necessary to guarantee that the S-adenosylmethionine pool was labeled at the time hydrazine and/or DNA was methylated, but it precluded the ability to use the specific activity of the S-adenosylmethionine pool to quantitate the levels of DNA methylation because the specific activity of that pool was constantly changing. An optical liquid

chromatographic technique which took advantage of the fluorescence of 7-methylguanine and O^6-methylguanine was developed to permit direct quantitation of DNA alkylation levels (Herron and Shank, 1979). This method has been refined repeatedly over the last three years and now permits detection of as little as 50 μmol 7-methylguanine/mol guanine (0.5 nmol per 20 mg DNA applied to the column) and 2 μmol O^6-methylguanine/ mol (4 pmol per 4 mg DNA applied to the column) in DNA using a fluorescence liquid chromatography detector rather than relying on radioassay.

Studies have been conducted to determine if the DNA methylation response is related to the dose of hydrazine administered. Doses of 30 to 90 mg hydrazine per kg body weight were used: 90 mg/kg approximates the LD_{50} from which animals die with severe liver damage 3 to 4 days after treatment; 60 mg/kg is approximately the LD_{001} as determined by extrapolation of dose-lethality data according to the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949). For the dose range of 45 to 75 mg hydrazine per kg body weight, the dose response curve was flat with levels of methylation between 400 and 500 μmol 7-methylguanine/mol guanine and 15-20 μmol O^6-methylguanine/mol guanine; at the LD_{50}, however, the methylation levels doubled to almost 900 μmol 7-methylguanine/mol guanine and 60 μmol O^6-methylguanine/mol guanine. Further studies at lower doses are required to determine whether DNA methylation is still detectable at exposures which do not produce cytotoxicity.

The kinetics of formation and persistence of 7-methylguanine and O^6-methylguanine in liver DNA following a single exposure to hydrazine have also been examined. The formation of these purines is rapid; the maximum level of 7-methylguanine in DNA is reached in only 30 minutes after oral administration of hydrazine, and the half-maximum level of O^6-methylguanine is reached in 45 minutes. Maximum alkylation levels are reached approximately 6 hours after hydrazine administration and remain constant during the subsequent 18 hours. 7-Methylguanine and O^6-methylguanine form concurrently at a ration of 10-20 7-methylguanine residues for every O^6-methylguanine molecule. The elimination rates for the two methylguanines from liver DNA are consistent with published levels for the removal of these bases from DNA in animals treated with such methylating intermediates as dimethylnitrosamine (Craddock, 1969; Pegg and Nui, 1978) methylnitrosourea (Kleihues and Margison, 1974), and 1,2-dimethylhydrazine (Herron and Shank, 1981). The half-life of 7-methylguanine in liver DNA in hydrazine-treated rats is approximately 47 hours, while O^6-methylguanine levels decrease more rapidly with an apparent half-life of 13 hours (Becker et al., 1981).

The regimen for the induction of liver cancer with hydrazine requires multiple exposures to the hepatotoxin (Severi and

Biancifiori, 1968). Thus liver DNA methylation patterns were examined in rats receiving multiple low doses of hydrazine. Daily oral doses of 3 mg hydrazine/kg body weight caused a 6.6% loss in body weight over a four-day period, and liver from animals treated 3 or 4 times appeared pale and fatty. 7-Methylguanine was just detectable (slightly greater than 50 µmol/mol guanine) by the third day in this pilot study. O^6-Methylguanine was not detected; in other experiments the molar ratio of 7-methylguanine to O^6-methylguanine was 10-20:1, and if present in the DNA from this study it presumably was below the limit of detection of the method used.

Preliminary studies have been done on hepatotoxins in addition to hydrazine to determine whether the DNA methylation process was specific to hydrazine toxicity or a non-specific response to hepatotoxicity in general (Barrows and Shank, 1981). Rats were given a single oral administration of carbon tetrachloride (1 g/kg body weight) or ethanol (5 g/kg body weight) and killed 12 hours later. Liver DNA was isolated, purified, and hydrolyzed, and analyzed by high pressure liquid chromatography with fluorescence monitoring. DNA from carbon tetrachloride-treated rats contained 560 µmol 7-methylguanine/mol guanine and 14 µmol O^6-methylguanine/mol guanine, a molar ratio of 40:1. Liver DNA from animals treated with ethanol contained 389 µmol 7-methylguanine/mol guanine but not detectable O^6-methylguanine, perhaps because the DNA sample size was small. Liver DNA from rats given 200 mg thioacetamine/kg body weight and 100 µCi (^{3}H-methyl)methionine hourly for 5 hours until decapitation contained trace amounts of 7-methylguanine and O^6-methylguanine as determined by radioassay of chromatographic eluents. Such results suggest that the DNA methylation response is not specific to hydrazine administration but may be a non-specific response to hepatotoxicity.

7-Methylguanine is a well-known constituent of RNA and is especially prevalent in transfer RNA (Dunn, 1963; Villa-Tervino and Magee, 1966); however, it is unlikely, for several reasons, that the DNA preparations analyzed in the above studies were contaminated with amounts of RNA sufficient to explain the claimed aberrancy in DNA methylation: 1) DNA from control animals was always free of detectable 7-methylguanine and O^6-methylguanine, testifying to the high purity of the DNA preparations; one might argue that in chemically injured liver, RNA may co-isolate with DNA, but this is unlikely as uridine was not detected when DNA from hydrazine-treated rats was digested enzymatically to release both purine and pyrimidine nucleosides. 2) Assuming the 7-methylguanine in DNA did represent contamination by RNA, and assuming that as much as 1% of all the guanine

bases in that RNA were methylated in the 7-position[1], then 2% of the DNA used in these studies would have had to be RNA to account for the 7-methylguanine detected, yet analysis of the DNA by the standard orcinol test failed to detect the presence of RNA, indicating the RNA content of the DNA preparation was less than 0.1%. 3) 1-Methyladenine, like 7-methylguanine, is also a known constituent of RNA (Dunn, 1963), yet its presence in the DNA preparations was never demonstrated even though the several high pressure liquid chromatographic methods employed were capable of detecting 1-methyladenine separately from 7-methylguanine and O^6-methylguanine (Herron and Shank, 1979). 4) Although 7-methylguanine is a known constituent of RNA, O^6-methylguanine is not, and thus its presence in damaged liver DNA cannot be explained by RNA contamination. It is concluded, then, that administration of the hepatotoxins, hydrazine, carbon tetrachloride, ethanol, and probably thioacetamide, resulted in methylation of liver DNA qualitatively, but not quantitatively, similar to the methylation of DNA seen after administration of such carcinogens as dimethylnitrosamine, 1,2-dimethylhydrazine, methylnitrosourea, etc.

Several mechanisms can be proposed to explain how the administration of a toxic dose of an inorganic hepatotoxin, such as hydrazine, can result in methylation of liver DNA guanine; four possible mechanisms are described:

1. Hydrazine may be metabolically methylated by S-adenosylmethionine to form monomethylhydrazine; monomethylhydrazine is known to be enzymatically oxidized to a compound capable of methylating liver DNA in vivo (Hawks and Magee, 1974). Several lines of evidence, however, suggest that monomethylhydrazine is not an important intermediate in hydrazine-induced DNA methylation. Rat liver DNA was found to be methylated to a greater extent in hydrazine-treated rats than in monomethylhydrazine-treated animals, following treatment with equitoxic doses of these chemicals; similar results from studies in mice given equimolar doses of the two compounds also failed to support monomethylhydrazine as an important intermediate in hydrazine-stimulated DNA methylation (Shank and Barrows, 1981). Moreover, in the present study, 15 minutes after hydrazine administration liver DNA contained appreciable quantities of 7-methylguanine and O^6-methylguanine; in fact, the time to half-maximum methylation levels was only 30-45 min. This methylation process would not be expected to occur so rapidly if hydrazine is first enzymatically methylated to monomethylhydrazine, and then the monomethylhydrazine is oxidatively metabolized to the ultimate

[1]Shank and Magee found that 0.06% of the total RNA guanine from normal Porton rat liver was methylated in the 7-position.

methylating species. These results, therefore, are not consistent with the suggestion that monomethylhydrazine is the more proximal methylating agent involved in hydrazine hepatotoxicity-induced liver DNA methylation.

2. The second hypothesis proposes that hydrazine administration may result in the loss of specificity of the normal DNA methylating enzyme. S-Adenosylmethionine-dependent DNA methyltransferase catalyzes the transfer of a methyl moiety from S-adenosylmethionine to the 5-position of cytosine in DNA of normal mammalian cells (Gold and Hurwitz, 1973); from one-half to two percent of DNA cytosine is methylated to 5-methylcytosine in vertebrates (Vanyushin et al., 1970). Hydrazine hepatotoxicity may alter the specificity of this DNA methyltransferase or the conformation of DNA substrate, or both, causing enzymatic DNA methylations at abnormal sites in DNA, such as the N-7 and O-6 atoms of guanine. Indeed, the 7-position of guanine closely resembles the 5-position of cytosine in the DNA helix. Both atoms extend into the major groove, and moreover, 90% of the 5-methylcytosine residues are found in the dinucleotide sequence 5'CpG (Wigler et al., 1981), such that the N-7 atom of guanine is in close proximity to normal DNA methyltransferase substrate. However, the O-6 atom of guanine, involved in base pairing in the DNA helix, does not closely resemble normal substrate for S-adenosylmethionine-dependent DNA methyltransferase. Radical alteration in substrate structure and/or DNA methyltransferase specificity would have to occur, presumably, in response to hydrazine hepatotoxicity to account for the formation of O^6-methylguanine by this mechanism.

3. A third hypothesis proposes that hydrazine administration may induce a direct chemical methylation of nucleophilic sites in DNA by S-adenosylmethionine. Concurrent formation of 7-methylguanine and O^6-methylguanine in both the dose-response and time-response experiments implies a similar mechanism of formation. Moreover, the molar ratio of 7-methylguanine to O^6-methylguanine approximately 20:1, resembles the ratio of these bases produced by the action of S_N1 methylating agents on nucleic acids in vivo and in vitro (Lawley and Shah, 1972). Since S-adenosylmethionine has been implicated as the proximal methyl donor in hydrazine-induced aberrant DNA methylation (Barrows and Shank, 1981), direct chemical methylation of DNA guanine by methonium ions generated unimolecularly from S-adenosylmethionine would be consistent with our results. Hydrazine hepatotoxicity may perturb the cellular distribution of S-adenosylmethionine, bringing this potential methylating agent in close proximity to DNA, where chemical methylation may occur. Recent work by Siemens and coworkers (1980) has demonstrated that small amounts of hydrazine added to cell cultures rapidly results in disorder in the cell membrane; such a structural upheaval within the cell by hydrazine might alter the intracellular distribution of S-adenosylmethionine.

4. Hydrazine administration could alter the control mechanisms regulating normal, highly dynamic DNA methylation. Recently, extracts from bacteria and mammalian cells have been shown to contain enzymatic activity capable of removing 7-methylguanine from DNA by catalyzing depurination of this base from the DNA polymer (Laval et al., 1981; Singer and Brent, 1981; Margison and Pegg, 1981). Several enzymes or proteins may be involved with the removal of O^6-methylguanine from DNA in vivo (Robins and Cairns, 1979; Karran et al., 1979; Pegg and Hui, 1978). Bacterial and mammalian cell extracts in vitro can remove O^6-methylguanine from DNA, although glycosylase activity is not found (Pegg, 1979). It has been suggested that these enzyme systems evolved in response to unusual biological DNA methylations (Singer and Brent, 1981), rather than in response to DNA adducts produced by chronologically more recent methylating carcinogens and mutagens. The apparent evolutionary conservation of enzymes capable of removing 7-methylguanine and O^6-methylguanine from DNA suggests that the underlying biochemical mechanisms associated with the formation of these products in DNA are also conserved, and further, that these apparently constitutive enzymes play an important role in cellular homeostasis. 5-Methylcytosine appears to be involved in gene regulation and differentiation (Razin and Riggs, 1980). 7-Methylguanine and O^6-methylguanine may also be normal consitutents of DNA, acting similarly to, but much more transiently than, 5-methylcytosine, such that highly transient methylation at these atoms in guanine may be related to expeditious gene function. The relative permanence of 5-methylcytosine in DNA suggests a function for this base more closely related to enduring gene regulation, i.e., differentiation. Hydrazine treatment in particular, and hepatotoxicity in general, may disrupt the balance between methylation and demethylation at the N-7 and O-6 atoms of guanine, affecting demethylation to a greater extent, leading to the accumulation of these purines in liver DNA.

Whatever the mechanism by which hydrazine administration results in methylation of liver DNA guanine moieties, the consequence of this response may represent a potential for heritable changes which may be important to the carcinogenicity of hydrazine.

A non-alkylating toxicant produces cell killing which is followed by a restorative hyperplasia; this hyperlasia takes place in an environment that may be abnormal and may be deficient in the normal regulatory feedback controls involved in homeostasis (Roberts, 1980). He suggested that erroneous DNA repair and chromosomal aberrations during the cellular regeneration may explain induction of cancers by chemicals which do not appear to directly modify the genetic material. Our recent finding of the presence of aberrant methylation in DNA following administration of a high dose of the inorganic toxicant, hydrazine, suggests that cytotoxicity may induce

DNA methylation and therefore may be qualitatively equivalent to administration of a strong alkylating agent; this may add another consideration to Roberts' hypothesis, but ultimately the effect is the same: replication of damaged DNA presumably resulting in a somatic mutation. If this effect is a result of administration of a cytotoxic dose of the non-alkylating toxicant, rather than due to some intrinsic property of the toxicant itself, then it may be that such compounds induce somatic mutations following only cytotoxic levels; lesser exposures which do not lead to DNA damage, cytotoxicity, and tissue regeneration would not be expected to lead to somatic mutation and cancer.

Some environmental chemicals increase the incidence of cancer in laboratory animals given maximum tolerated doses over half a lifetime; in such studies chloroform has been associated with cancer of the liver in mice (Eschenbrenner, 1945; Page and Saffiotti, 1976) and kidney in rats (Page and Saffiotti, 1976), formaldehyde has been associated with cancer in the nasal turbinates (Swenberg et al., 1980), and hydrazine has been associated with cancer of the liver, lung and nasal turbinates in rats (Severi and Biancifiori, 1968; MacEwen et al., 1979), and liver and lung in mice (Severi and Biancifiori, 1968; Biancifiori and Ribacchi, 1962; Yamamoto and Weisburger, 1970). Within the limits of a bioassay vinylidine chloride seems to be carcinogenic in the mouse only when sufficient amounts of the compound have been given so that toxic damage to the kidney results. In studies by McKenna and coworkers (1977) and Reitz and colleagues (1980) kidney tumors were found in mice as long as levels of vinylidene chloride were high enough to produce toxic injury to renal tissue; as doses fell below cytotoxic levels, kidney tumors failed to appear even though sufficient numbers of animals were used to detect expected tumor incidences based on linear extrapolation from dose-response curves. These results seem to be explained in part by overloading the biochemical detoxication mechanisms, in this instance depleting the stores of reduced glutathione, resulting in cytotoxicity and presumably the restorative hyperplasia in an abnormal environment to which Roberts (1980) referred.

The observation that hydrazine toxicity results in the aberrant methylation of liver DNA may be highly relevant to the argument that cytotoxicity can be causally associated with carcinogenesis. If this phenomenon is a general response to toxicity, understanding the mechanism by which the methylation occurs could assist in the interpretation of results from carcinogenicity bioassays in which increased cancer incidences are observed only at or near cytotoxic levels of the test compound.

The oral administration of hydrazine to rats and mice results in the extremely rapid methylation of liver DNA, and one hypothesis has been proposed that this may represent not aberrant methylation of the DNA but rather an inhibition of normal demethylation of DNA. This proposed mechanism presumes that there are sites of normal methylation on DNA in addition to the 5-position of cytosine. Many investigators have tried to detect other sites of methylation in mammalian DNA and have had no success. Researchers (Gold et al., 1963; Shank and Magee, 1967; Swann et al., 1971; Craddock, 1971) actively following the consequences of the formation of 7-methylguanine in DNA after exposure to carcinogenic methylating intermediates have made valiant efforts to find 7-methylguanine in normal mammalian DNA and have come to the conclusion that if such a base is present, it is present at extremely low concentrations. It may also be possible that such a base could be present in mammalian DNA not necessarily at such low concentrations but for such a short residence time that radiolabel pulse studies are not capable of detecting the bases. The observation of these additional, highly transient methylated bases in liver DNA after hydrazine administration may possibly be explained by the induced toxicity interfering with the active demethylation of the DNA. This explanation in turn presumes that there are constitutive mechanisms for the rapid removal of these methylated bases. These studies are expected to contribute to the understanding between cytotoxicity and carcinogenesis and may also make a contribution to the structure and function of DNA.

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